

REVIEW



## Approaches to inhibiting oncogenic K-Ras

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### ABSTRACT

Activating somatic K-Ras mutations are associated with >15% all human tumors and up to 90% of specific tumor types such as pancreatic cancer. Successfully inhibiting abnormal K-Ras signaling would therefore be a game changer in cancer therapy. However, K-Ras has long been considered an undruggable target for various reasons. This view is now changing by the discovery of allosteric inhibitors that directly target K-Ras and inhibit its functions, and by the identification of new mechanisms to dislodge it from the plasma membrane and thereby abrogate its cellular activities. In this review, we will discuss recent progresses and challenges to inhibiting aberrant K-Ras functions by these two approaches. We will also provide a broad overview of other approaches such as inhibition of K-Ras effectors, and offer a brief perspective on the way forward.

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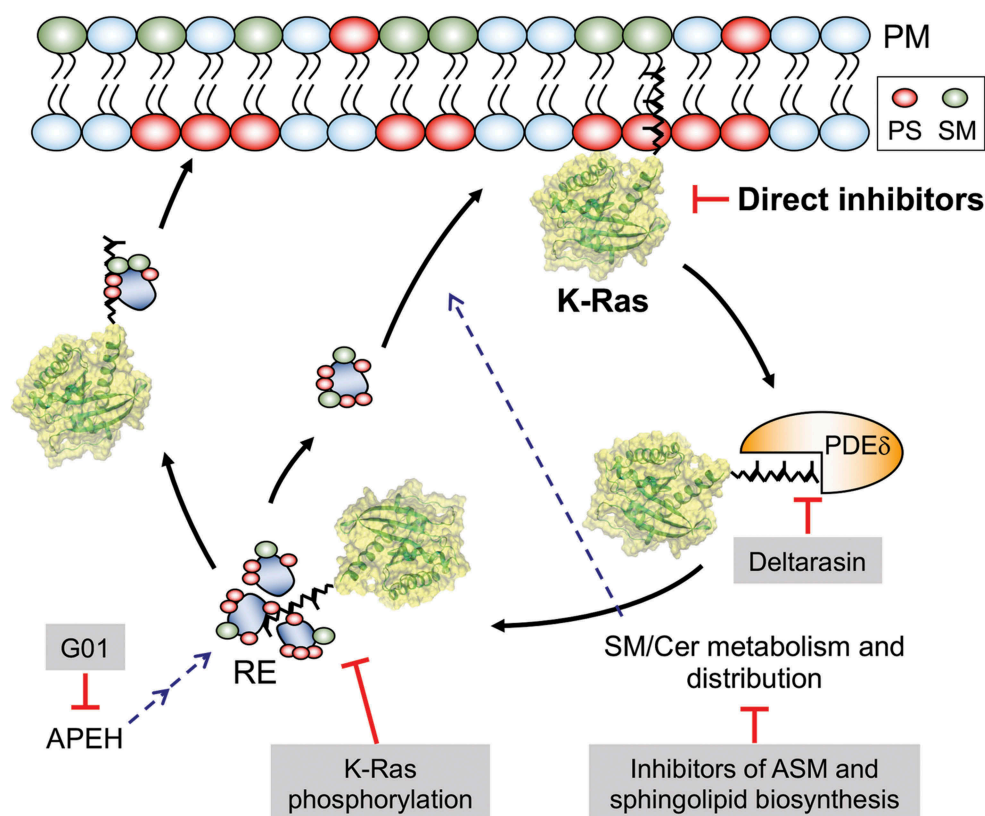
## Introduction

Ras proteins are plasma membrane (PM)-associated molecular switches that oscillate between GDP-bound inactive and GTP-bound active conformational states [1] to regulate a variety of signaling pathways crucial for cell growth and differentiation [2]. The three major Ras isoforms in humans (H-Ras, N-Ras and K-Ras) share 95% sequence identity at their catalytic domain (residues 1–166) but diverge at their C-terminal 21/22 residues that harbor a lipid-modified membrane targeting motif [3–6]. The catalytic domain consists of lobe1 (residues 1–86) and lobe2 (residues 87–166) [6]. The nucleotide binding and effector-interacting switch 1 (residues 30–38) and switch 2 (residues 59–76) regions are located in lobe1 and undergo major conformational changes upon GDP/GTP exchange and GTP hydrolysis. Nucleotide exchange is facilitated by guanine nucleotide exchange factors (GEFs) such as son of sevenless and GTP hydrolysis is catalysed by GTPase activating proteins (GAPs) such as neurofibromin. Defective GTPase activity of Ras due to somatic mutations, typically at codons 12, 13 and 61, is associated with 15–20% of all human cancers, and K-Ras mutations account for 85% of all Ras mutations [4,7,8]. It is thus clear that a drug that selectively inhibits K-Ras would be a game changer in cancer therapy.

Ras proteins primarily localize to the inner leaflet of the PM in order to transduce extracellular signals to the nucleus [9,10]. For high affinity PM binding, Ras proteins undergo a series of post-translational modifications at the C-terminal CAAX motif (where C = Cys, A = aliphatic amino acids, and X = Met or Ser). First, a cytosolic farnesyltransferase attaches a farnesyl group to the Cys, which allows Ras to attach to the cytosolic leaflet of the ER [11,12]. RCE1 (Ras converting CAAX endopeptidase 1) then removes the AAX tripeptide, followed by the methylation of the now C-terminal prenylated Cys by ICMT (isoprenylcysteine carboxyl methyltransferase) [12,13]. N-, H-, and K-Ras4A (the alternative splicing variant of K-Ras) are further modified with the addition of palmitic acids on one or two other Cys residues near the prenylated Cys [11], allowing Ras to interact with and localize to the PM. K-Ras4B (hereafter, K-Ras) is unique in that it has a single farnesyl chain preceded by a polybasic domain of six Lys residues [14]. The strong positive charge of this polybasic domain allows K-Ras to interact with anionic phospholipids in the PM through electrostatic interaction [15,16].

Broadly, four alternative approaches of developing therapies for K-Ras-driven cancers are being pursued: 1) dissociation of K-Ras from the PM; 2) direct allosteric

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**Figure 1. Schematics showing promising approaches to inhibiting oncogenic K-Ras:** K-Ras activity can be inhibited directly or by agents that facilitate its dissociation from the plasma member. PDE $\delta$  binds to the PM-dissociated K-Ras and unloads it in the perinuclear region, whence K-Ras is translocated to the PtdSer- and SM-enriched recycling endosome (RE) for redelivery to the PM by vesicular transport. Disrupting its interaction with PDE $\delta$  or RE reduces the concentration of K-Ras at the PM. Also, perturbation of SM/Cer metabolism and distribution, which regulates PM PtdSer content, depletes PtdSer of the PM, resulting in K-Ras PM dissociation. Dysregulating RE activity by APEH inhibition further results in the mislocalization of PtdSer and K-Ras from the PM. PS – phosphatidylserine, SM – sphingomyelin, Cer – ceramide, ASM – acid sphingomyelinase, APEH – acylpeptide hydrolase, PDE $\delta$  – phosphodiesterase  $\delta$ , G01 – a synthetic small molecule inhibitor of APEH.

inhibition of K-Ras; 3) inhibition of K-Ras downstream effectors; and 4) dysregulation of cell metabolism. In this review, we will focus on the first two approaches and discuss in detail recent progresses and challenges to inhibiting aberrant K-Ras functions by small molecule inhibitors that dislodge it from the PM or directly bind to K-Ras and allosterically modulate its biochemical activities (Figure 1). We will then provide an overview of the latter two approaches, and conclude with a brief perspective.

### Dissociation of K-Ras from the PM

The first attempt at blocking the interaction of Ras with the PM was via the inhibition of farnesylation by farnesyltransferase inhibitors (FTIs). FTIs were highly effective in cell culture and mouse models of H-Ras tumors, but failed in K-Ras tumors because of geranylgeranylation, an alternative prenylation pathway that effectively

subverted the intended therapeutic mechanism [17]. Despite the clinical failure of FTIs as an anti-K-Ras drugs, inhibition of PM interaction remains a valid therapeutic approach to abrogating K-Ras oncogenic activity. Recent studies identified new molecular mechanisms that regulate K-Ras PM interaction and its signaling: depleting the phosphatidylserine (PtdSer) content of the PM, enhancing K-Ras phosphorylation, and disrupting K-Ras interaction with its chaperone protein phosphodiesterase (PDE $\delta$ ).

### PM PtdSer depletion by perturbing sphingomyelin metabolism

PtdSer is asymmetrically concentrated on the inner leaflet of the PM and is responsible for the significant negative electrostatic potential of the PM [18]. K-Ras interacts with the PM through the combined effects of the polybasic domain and the farnesyl chain of the lipid

anchor, which provides high selectivity for PtdSer over other anionic phospholipids [15]. Depletion of PtdSer mislocalizes K-Ras from the PM and blocks K-Ras signaling by disrupting nanoclusters, an essential Ras signaling platform [16,19,20]. Recent studies have shown that sphingomyelin (SM) metabolism modulates K-Ras PM localization by regulating the PtdSer content of the PM, thus opening new opportunities for inhibiting K-Ras.

Two classes of inhibitors that modulate SM metabolism have been reported (Figure 1). The first is inhibitors of acid sphingomyelinase (ASM), which converts SM to ceramide in the lysosome. It was found that fendiline, a potent inhibitor of ASM redistributes PtdSer and K-Ras (but not H-Ras) from the PM to endomembranes [21,22]. Supplementation with exogenous PtdSer restores K-Ras PM interaction in fendiline-treated cells, suggesting that the K-Ras PM mislocalization is through PtdSer depletion at the inner PM leaflet [21]. Fendiline also reduces cellular ceramide level and induces SM endosomal accumulation. Supplementation with recombinant ASM or exogenous ceramide restores K-Ras and PtdSer back to the PM in fendiline-treated cells, suggesting that ASM-mediated cellular balance of SM/ceramide regulates PtdSer localization at the PM, resulting in K-Ras PM interaction [21]. A wide variety of ASM inhibitors including tricyclic antidepressants are also shown to deplete PM PtdSer and mislocalize K-Ras from the PM [23]. Consistent with this, K-Ras is shown to be mislocalized from the PM in patient-derived Niemann-Pick type A and B cell lines [21]; Niemann-Pick type A and B diseases are lysosomal storage disorders caused by inactivating and partial-loss-of-function mutations, respectively, in the *SMPD1* gene that encodes ASM [24]. ASM inhibitors disrupt oncogenic K-Ras signaling and its PM nanoclusters, and block the growth of a range of human cancer cells expressing oncogenic mutant K-Ras but not wild-type K-Ras [22,23,25]. Taken together, these studies identify ASM as an attractive target for the development of anti-K-Ras therapies.

The second group of inhibitors that modulate SM metabolism target enzymes involved in sphingomyelin biosynthesis. Perturbing the cellular SM/ceramide balance by dysregulating enzymes involved in sphingomyelin biosynthesis is shown to disrupt K-Ras PM interaction and its signal output. In a genetic study using RNA interference against *C. elegans* genes encoding enzymes in the SM/ceramide biosynthesis pathway, the authors found that knockdown of 14 enzymes suppresses the LET-60 G13D (a K-RasG13D ortholog in *C. elegans*)-induced multivulva phenotype [23]. Furthermore, pharmacological agents targeting these

enzymes in mammalian cells deplete PtdSer and mislocalize K-Ras from the PM [23]. These compounds also disrupt the K-Ras nanoclustering and inhibit the proliferation of pancreatic cancer cell lines expressing oncogenic mutant K-Ras [23]. Although these pharmacological agents either increase or decrease cellular SM levels, they all perturb cellular SM distribution. Based on these observations, the authors proposed that a correct cellular distribution of SM at appropriate concentrations is required for maintaining PtdSer and K-Ras at the PM, and that pharmacological tools targeting the sphingolipid pathways may provide novel therapeutic strategies for the treatment of K-Ras-dependent cancers [23,26].

### ***PM PtdSer depletion by perturbing recycling endosomal activity***

A recent study identified acylpeptide hydrolase (APEH) as a new novel protein that regulates K-Ras and PtdSer PM localization. APEH is a ubiquitously expressed cytosolic enzyme that catalyses the removal of N-acylated amino acids from acetylated peptides, and it is involved in the ubiquitin-proteasome protein degradation machinery [27,28]. APEH knockdown or inhibition mislocalizes K-Ras and PtdSer from the PM, which is rescued by ectopic expression of APEH [29]. The study found that APEH-mediated PM mislocalization of K-Ras and PtdSer depends on suppression of recycling endosome (RE) function. PtdSer is the most abundant lipid in REs among intracellular organelles [30], and perturbation in endosomal sorting of PtdSer results in the PM PtdSer depletion and K-Ras PM mislocalization [31]. Also, the RE operation in concert with PDE6 $\delta$  and Arl-2/3 maintains K-Ras at the PM (discussed in detail later) [32,33]. The authors suggest that the failure to maintain PM PtdSer content in APEH-knockdown cells is at least in part due to aberrant RE function. Furthermore, APEH knockdown or inhibition abrogates Ras/MAPK signaling in cells expressing oncogenic mutant K-Ras and inhibits the growth of K-Ras-positive cancer cells [29,34]. Taken together, this study proposes that perturbation of RE activity could be an attractive approach for inhibiting K-Ras activity, and that APEH is a novel drug target for a potential anti-K-Ras therapeutic.

### ***Enhancing K-Ras phosphorylation***

Protein kinase C (PKC) directly phosphorylates K-Ras at Ser181 and to a lesser extent at Ser171 and Thr183, redistributing K-Ras from the PM to the endomembranes and mitochondria, triggering enhanced apoptosis [35]. PKC

activators can further suppress the growth of K-Ras tumors in nude mice by stimulating K-Ras phosphorylation [35,36], suggesting that K-Ras phosphorylation is a valid target for blocking oncogenic K-Ras signaling. A recent study identified cyclic GMP-dependent protein kinase 2 (PKG2) as a new K-Ras kinase that phosphorylates K-Ras at Ser181 in response to the AMPK-eNOS-sGC-PKG2 pathway activation [33] (Figure 1). Using pharmacological agents targeting enzymes in the pathway, it was found that direct or indirect activation of AMP-activated protein kinase (AMPK) stimulates the activity of endothelial nitric oxide synthase (eNOS), one of the AMPK downstream effectors. This in turn elevates cellular NO levels, which promotes soluble guanylyl cyclase (sGC) activity, generating cGMP from GTP. Activated PKG2, but not PKG1, is recruited to the PM and phosphorylates K-Ras at Ser181 by the elevated cGMP. Unlike PKC, which acutely mislocalizes K-Ras from the PM after phosphorylation, K-Ras is mislocalized from the PM at  $t_{1/2} = 40\text{min}$  after PKG-mediated phosphorylation. The authors proposed that phosphorylated K-Ras by PKG is not instantly dissociated from the PM, but rather progressively lost via endocytic recycling [33].

Stimulation of the AMPK-eNOS-sGC-PKG pathway shows anti-cancer response. Chronic treatment with pharmacological agents that activate components in the AMPK-eNOS-sGC-PKG inhibits the growth of non-small cell lung cancer cells expressing oncogenic mutant K-Ras [33]. Also, metformin, an antidiabetic drug that activates AMPK through lowering cellular ATP levels [37], is reported to be associated with a 31% reduction in cancer risk in a meta-analysis of 5 observational studies of all cancer types [38]. It also showed inhibitory activity in tumor growth in preclinical endometrial cancer models, with the greatest response being in cells expressing oncogenic mutant K-Ras [39]. Another activator of the pathway is sildenafil also known as Viagra, which inhibits PDE5 to elevate cellular cGMP levels and thereby activate PKG. Oral administration of sildenafil suppresses colorectal cancer in mice induced by the potent carcinogens azoxymethane/dextran sulfate sodium [40]. Furthermore, PKG2-null mice develop crypt hyperplasia in the colonic epithelium, while ectopic PKG2 expression in colorectal cancer cell lines inhibits proliferation [41]. In contrast, some components of the AMPK-eNOS-PKG pathway may promote tumorigenesis. For example, advanced PDACs with a genetic deficiency of eNOS or treatment of mice with a NOS inhibitor suppresses the development of preinvasive pancreatic lesions and shows a tendency toward an extended lifespan [42]. Also, acute PKG activation in vascular smooth muscle cells enhances MAPK signaling [43]. One explanation for the inconsistency in the anti-cancer response of the pathway is the extent of

K-Ras phosphorylation. Using computational modeling, biochemistry and electron microscopy techniques, it has been shown that PKG2-mediated K-Ras phosphorylation acutely increases both phosphatidylinositol 3-kinase/Akt and Raf/MAPK activation by altering K-Ras PM nano-clustering. This is attenuated by a progressive loss of phosphorylated K-Ras from the PM, which subsequently abrogates K-Ras signaling [33]. Based on these data, it is possible that in certain tissues, AMPK-eNOS-PKG signaling is sufficient to phosphorylate K-Ras but not enough to remove it from the PM, resulting in elevated K-Ras signal output. Clearly, more work is required to fully establish the therapeutic potential of K-Ras phosphorylation. In particular, further characterization of cancer types sensitive to the pharmacological activators of the AMPK-eNOS-sGC-PKG pathway will need to be elucidated, and the long-term beneficial effects of the activators need to be analysed in the context of K-Ras-positive cancers.

### ***Inhibiting K-Ras interaction with its chaperone protein***

Maintenance of K-Ras at the PM requires the activities of the chaperone protein PDE6 $\delta$ . The non-catalytic  $\delta$  subunit of PDE6 binds to endocytosed K-Ras via the farnesyl tail and releases it to the perinuclear membrane in an Arl2-dependent manner. K-Ras then electrostatically interacts with the RE and returns to the PM [32,44,45]. Disrupting the interaction of K-Ras/PDE6 $\delta$  by PDE6 $\delta$  knockdown or a PDE6 $\delta$  inhibitor, deltarasin, mislocalizes K-Ras from the PM [32,46]. Deltarasin blocks the interaction by binding to a hydrophobic pocket of PDE6 $\delta$ , to which the farnesyl group of K-Ras would bind [46]. PDE6 $\delta$  inhibition further shows anti-K-Ras activity in human cancer cells. Genetic or pharmacologic inhibition of PDE6 $\delta$  blocks proliferation and survival of colorectal cancer cells expressing oncogenic mutant K-Ras, whereas the growth of isogenic cell lines in which the oncogenic K-Ras has been removed, or cell lines with oncogenic mutant B-Raf or EGFR overexpression are not affected by PDE6 $\delta$  inhibition [47]. Deltarasin treatment also inhibits the growth of pancreatic cancer cells expressing oncogenic mutant K-Ras *in vitro* and *in vivo* [46]. In addition to inhibiting K-Ras signal output by blocking K-Ras/PDE6 $\delta$  interaction, deltarasin has K-Ras-independent effects. Deltarasin also elevates autophagy through activating the AMPK-mTOR pathway [48], and autophagy inhibition in deltarasin-treated cancer cells potentiates deltarasin-mediated cell death. This led the authors to propose that deltarasin therapy in

combination with an autophagy inhibitor can be a good strategy for treating K-Ras-driven cancers [48].

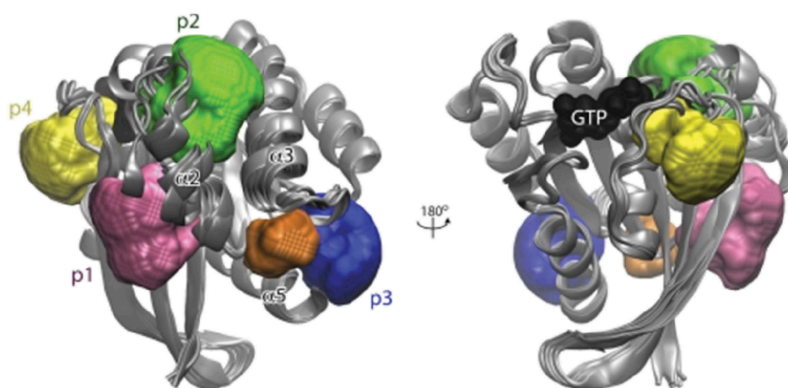
Despite the reported anti-cancer activities of deltarasin, it is unclear whether the effects are specifically through K-Ras inhibition. PDE6 $\delta$  binds to many farnesylated Ras superfamily members including H-Ras, Rheb, and Arl2/3 [45,49], which may account for the elevated autophagy in deltarasin-treated cells. Furthermore, K-Ras knockout in mice is embryonic lethal, whereas PDE6 $\delta$  knockout mice are viable and fertile [50,51], suggesting that K-Ras is functional in the absence of PDE6 $\delta$ . Taken together, although the effects of PDE6 $\delta$  inhibition by deltarasin in K-Ras-driven cancers are exciting, translation into the clinic may require further characterization of its K-Ras specificity.

### Direct inhibition of K-Ras

As described in section 1, considerable effort has been devoted toward indirectly inhibiting aberrant K-Ras functions, such as by dislodging it from the PM [22,31] or inhibiting its partner proteins [46,52]. This was because direct inhibition has been deemed impossible in part because Ras has high (picomolar) affinity for GDP and GTP that exist at high ( $\sim 0.5$  mM) concentration in the cell [7,53]. Another challenge is the conservation of the nucleotide-binding pocket among a diverse group of small GTPases with unrelated functions [1,54]. These issues made competitive inhibition of K-Ras impractical and avoiding off-target effects difficult. In principle, many of these challenges could be overcome by allosteric inhibitors, but first it was necessary to establish that that Ras is an allosteric enzyme. The first clue about the allosteric nature of

Ras emerged from molecular dynamics (MD) simulation studies of H-Ras in a simplified model membrane [55]. Additional studies of Ras dynamics in solution and when membrane-bound (reviewed in [3,5,56]), first led to the recognition that Ras has two lobes engaged in long-range coupled motions [6] and then the suggestion that Ras is potentially druggable by allosteric mechanisms [57]. The concept of Ras allostery was initially somewhat controversial given its small size and shallow surface that lacks any obvious ligand binding site outside of the canonical nucleotide binding site. This has changed by the identification of up to four allosteric ligand binding sites first using computational approaches [58–64], and then using NMR or crystallographic studies of ligands bound to these pockets [65–68] (Figure 2). Many ligands that directly bind to the allosteric sites of K-Ras and modulate its functions have been reported [58,63,65,69,70], including small-molecules [58,70,71], peptidomimetics [72,73], monobodies [74], and even DARPinS [75,76].

To our knowledge, none of the reported non-covalent K-Ras inhibitors have made it to clinical trial. In this regard, covalent inhibitors including GDP analogues [77] or other small-molecules [65] targeting G12C K-Ras may have a better chance of eventual success [78], but their application is likely limited to a few cancer types such as small cell lung cancer [79]. Therefore, non-covalent allosteric inhibition will still be required to target many critical K-Ras mutations including G12D, G12V, G13D and Q61H which together account for >78% of all K-Ras-associated cancers [4,79]. While the examples cited above highlight the vulnerabilities of K-Ras, no approach seems to emerge that is capable of identifying sufficiently potent and selective inhibitors that overcome the persistent problems of weak affinity and pan-Ras activity (see



**Figure 2. Druggable allosteric pockets on the catalytic domain of K-Ras.** The sites most frequently targeted by published small molecules are pocket p1 near the core beta-sheet (pink) and pocket p2 between switch 2 and helix 3 (green). Pocket p3 near the C-terminus (blue) and pocket p4 behind switch 1 (yellow) are somewhat shallow and more polar than p1 and p2. See Grant et al. [63], for a detailed structural analysis of the four pockets and Gupta et al. [84], for a comparison of their druggability profile. Image reproduced from Grant et al. [63].

refs [80–83] for recent examples). The solution may lie in ensemble-based approaches, considering the highly dynamic nature of K-Ras. Indeed, a recent report found a ~ 10% success rate of predicted-to-confirmed K-Ras binders using this approach [84]. A similar approach yielded a highly promising pyrazolopyrimidine-based lead compound that appears to be selective toward GTP-bound K-Ras and disrupts effector binding and reduces signal transduction through mutant K-Ras [85].

Recent reports on K-Ras dimerization and clustering [84,86–94] opened up alternative ways of thinking about Ras inhibition, such as preventing dimer formation as exemplified by two recent reports [76,95,96]. Another potentially fertile area of intervention is the concept of membrane reorientation, which was originally proposed based on simulations and then verified experimentally [55,97–103]. Future studies may discover inhibitors that target the inter-switch pocket and stabilize a membrane orientation state that is incapable of effector interaction, as found in two recent reports [104,105].

## Other approaches

Arguably the most extensively studied approach of indirectly inhibiting Ras functions is the inhibition of its downstream effectors. These targets contribute to Ras-dependent cancer initiation and/or maintenance. A number of promising inhibitors targeting a variety of Ras effectors have been developed and tested in clinical trials. A key limitation of this approach is that inhibition of one Ras effector pathway can be compensated for by other Ras downstream effectors, and inhibition of multiple Ras signaling pathways are lethal for normal cells, resulting in high toxicity [7]. One example is the B-Raf-specific inhibitors. These inhibitors produce excellent responses in patients with B-Raf mutant melanoma [106], but patients develop resistance because the inhibitors paradoxically activate the MAPK cascade in melanoma cells expressing oncogenic mutant N- or K-Ras via a mechanism that involves C-Raf hyperactivation [20,107,108]. A somewhat related approach is to inhibit proteins that have synthetic lethal interaction with oncogenic K-Ras; i.e., inhibiting proteins whose loss of function is lethal only in the presence of oncogenic K-Ras. This approach has been inspired most strongly by the successful use of PARP inhibitors in the clinic to treat BRCA-defective cancers [109]. Genome-wide RNA interference screenings identified several genes required for the survival of cancer cells harboring oncogenic mutant K-Ras [109]. However, the screening data showed lack of overlap between the results, with the possible exception of proteasome components [109], and pharmacological inhibitors of the identified genes have not been proven as targeting cancer cells harboring oncogenic K-Ras [110].

Another approach to targeting K-Ras-driven cancers is dysregulation of cell metabolism. Cancer cells alter their metabolism to meet the increased energy requirement for their growth, and oncogenic K-Ras promotes such metabolic rewiring, although the specifics may differ depending on tumor type and genetic context [111,112]. In pancreatic cancer cells that are K-Ras- and autophagy-dependent for their growth, oncogenic K-Ras/MAPK signaling upregulates autophagy in part by impairing other K-Ras- or MAPK-driven metabolic processes [113], and concomitant inhibition of K-Ras/MAPK and autophagy synergistically inhibits tumor growth [113–115]. A clinical trial (NCT03825289) has been initiated recently to test the efficacy of a combined therapy of MEK inhibitors with hydroxychloroquine, an autophagy inhibitor.

## Perspectives

K-Ras has been considered as undruggable for many years despite its critical role in many human cancers. However, recent intensive studies identified a number of vulnerabilities that rendered K-Ras druggable. In this review, we have focused on two approaches that we believe are most promising: dislodging K-Ras from the PM and directly targeting its catalytic domain with small molecule allosteric inhibitors. Although K-Ras is localized primarily at the PM for stimulating its downstream effectors, the mechanisms of its trafficking to and maintenance at the PM are not fully elucidated. A better understanding of these processes is beginning to provide valuable insights into novel approaches of developing anti-K-Ras therapeutics. Furthermore, since the dynamics of K-Ras differs when it is in solution and membrane-bound, approaches that account for these differences may yield novel lead compounds with desirable modes of action such as disrupting effector binding. Focus on allosteric instead of competitive inhibition and emphasis on dynamics where Ras isoforms diverge might also allow for the discovery of K-Ras-selective inhibitors.

Combination therapy for cancer treatment is a well-established practice. With only a few exceptions, cytotoxic cancer chemotherapy is most effective when applied as a concurrent treatment of a cocktail of drugs with different mechanisms of action. It would be intriguing to examine the effects of combining K-Ras inhibitors that target different aspects of its activities. Ultimately no single inhibitor, even one that is K-Ras selective, may treat all of the many types of K-Ras-driven cancers, and different mutations drive different cancers. That said, the ability to treat any one of these cancers would itself

be a major breakthrough, and will open opportunities for rational re-design and combination therapies until a truly personalized therapy targeting a specific K-Ras mutation becomes a reality.

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